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BINDING MOLECULES 1

It was disclosed in patent application PCT/GB91/01134 that antibody fragments can be displayed on the surface of bacteriophage fd and that they will bind antigen. Antibody fragments can be directly selected using this characteristic. This ability to isolate antibody fragments (Fab, Fv, scFv and VH) using their display on the surface of filamentous bacteriophage has opened up the prospect of the isolation of antibody specificities (i.e. antibodies directed against a particular antigen) that were difficult or impossible to isolate previously. In particular PCT/GB91/01134 demonstrates that antibody specificities can be isolated from a human who has not been specifically immunised ('unimmunised'), even specificities for antigens such as 2-phenyl-5-oxazolone to which humans will not normally be exposed. In contrast, screening of a bacteriophage lambda library (where no analogous selection procedure is available) failed to isolate a single antibody against tetanus toxoid from an unimmunised human donor (M.A.A. Persson et al Proc.Natl. Acad.Sci.U.S.A 88 2432-2436, 1991). Antibodies of high specificity have been obtained from phage fd libraries of 'natural' antibodies from unimmunised donors, for instance an antibody against turkey egg lysozyme was isolated with a dissociation constant of 87nM for turkey egg lysozyme which did not cross react with hen egg lysozyme (see example 1). The possibility therefore exists of the isolation of antibodies to any antigen from a phage library which is sufficiently large and diverse.

A preferred source for the generation of diverse libraries is IgMmRNA. It is was found in example 43 of PCT/GB91/01134 that antibody fragments directed against turkey egg lysozyme and 2-phenyl-5-oxazolone were much more readily isolated from a phage library derived from the IgM mRNA from an unimmunised human donor, than from one prepared from IgG mRNA. Furthermore, no 2-phenyl-5-oxazolone binding antibody fragments could be isolated from a library of 2000000 phage antibody clones prepared from IgGmRNA of unimmunised mice (T.Clackson et al, Nature 352 624-628.1991).

Antibody specificities can be isolated from these libraries of 'natural' human antibodies which are directed against non-self antigens of human origin, for instance specificities directed against human blood group B from libraries prepared from subjects of blood group O.

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It is a demanding task to isolate an antibody fragment with specificity against self antigen. Animals do not normally produce antibodies to self antigens, a phenomenon called tolerance. Autoimmune diseases may result from a breakdown in tolerance. In general, vaccination with a self antigen does not result in production of circulating antibodies. It is therefore difficult to raise antibodies to self antigens, particularly in humans. It is possible to raise antibodies that recognise human antigens in other animals, especially if the epitope is not too closely related between the two animals. If a human antibody is then required it is necessary to 'humanise' the antibody by CDR grafting (patent GB2188638B). Phage antibody technology (PCT/GB91/01134) offers the ability to isolate such human antibodies directly. In this application, we demonstrate that antibodies against self-antigens can be isolated from phage libraries derived from nonimmunised humans.

In examples 1 to 3 and in PCT/GB91/01134 we have used fusion of antibody fragments to gene 3 protein of filamentous bacteriophage for display and selection of antibody fragments derived from unimmunised humans. An alternative approach would be to fuse antibody fragments to gene 8 protein or other surface molecules of filamentous bacteriophage.

The simplest anti-self antibodies to isolate should be those from patients with significant concentrations of circulating autoantibodies, due to a breakdown in the tolerance mechanism. The antibodies prepared from V gene libraries will then derive from the mRNA of plasma cells secreting IgG (or IgM) antibody. For instance, anti-self antibodies might be isolated from patients with autoimmune diseases, for example anti-acetylcholine receptor antibodies would be expected to be isolated from antibody repertoires made from the IgG mRNA of myasthenia gravis patients. For example, an antibody fragment specific for human thyroid peroxidase has been isolated from a bacteriophage lambda library from a patient with thyroid autoimmune disease (S.Portolano et al Biochem. Biophys. Res. Commun. 179 372-377, 1991). This however required extensive screening of 200,000 plaques to obtain one clone. In addition, this library was derived from thyroid tissue, a procedure not readily applicable in most instances. In contrast, the power of selection available using the phage system, demonstrated in PCT/GB91/01134 allows the ready

isolation of autoantibodies from the IgM mRNA of peripheral blood lymphocytes of a donor without disease. We show in example 3 that antibodies binding to human thyroglobulin (which are found in the sera of many people without autoimmune disease), can be isolated from phage repertoires prepared from unimmunised humans.

However, the vast majority of self antigens do not have associated circulating autoantibodies. In this application, we demonstrate that even antibodies against human tumour necrosis factor-α (a self antigen only rarely having autoantibodies directed against it) can be isolated as described in example 2 from the same library as the antibodies directed against thyroglobulin. This opens the prospect of the direct isolation of human antibodies binding to human antigens for a number of purposes such as antibodies which bind to circulating hormones to block, modify or potentiate their action or antibodies that bind to cell surface antigen for imaging or killing for example of cancer cells.

The origin of the V genes that contribute to anti-self antibodies isolated from phage display libraries is not clear.Tolerance to self antigens by the immune system (preventing the generation of antibodies directed against them) is mediated by either clonal deletion or functional inactivation (anergy) of self-reactive B lymphocytes (D.A.Nemazee & K.Burki Nature 337 562-566, 1989; C.C.Goodnow et al Nature 334 676-682, 1988; S.B.Hartley et al Nature 353 765-769, 1991; D.M.Russell et al Nature 354 308-311, 1991). In either case little circulating anti-self antibody is detectable for most antigens. However, in the case of anergy, functionally inactivated self-reactive cells from the B cell lineage persist in peripheral lymphoid organs leading to B cells in circulation. These rare lymphocytes with anti-self specificity may provide heavy or light chain partners (or even both) for phage antibodies with anti-self specificities. Alternatively, such anti-self specificities may arise from the combination in the library of a VH domain with a VL domain to give a specificity that is normally deleted if it occurs in nature. For this reason, combinatorial libraries and 'chain-shuffled' libraries such as those described in patent applications PCT/GB91/01134 and 9120252.3 may be a particularly rich source of anti-self antibodies. Whichever of these possibilities applies, a selection procedure of great power, such as that provided by phage antibodies, is required to obtain such rare anti-self antibodies.

This application shows that anti-self antibodies can be isolated from libraries prepared using mRNA derived from peripheral

blood lymphocytes. Other sources of such anti-self antibodies may be fetal mRNA or cord blood mRNA (P.M.Lydyard et al Scand J Immunol 31 33-43, 1990) or libraries prepared by the synthetic recombination of V, D and J segments.

Phage libraries may be prepared which are enriched for antibodies directed against self. B lymphocytes express surface IgM and surface IgD before stimulation with antigen but express little soluble IgM or IgD. These unstimulated cells are more likely to contain antibody genes with anti-self specificities.In contrast, terminally differentiated plasma cells which secrete soluble antibodies express little surface immunoglobulin. The preparation of cDNA for phage library preparation using primers which are specific for surface IgM or surface IgD will produce a repertoire of antibody genes enriched for the naive, unselected genes. In B lymphocytes which have been functionally silenced by exposure to self there are greatly reduced levels of surface IgM but unchanged levels of surface IgD (C.C.Goodnow et al. supra). Hence, a primer specific for surface IgD may be particularly suitable for isolation of anti-self antibodies.
There is some evidence that B lymphocytes directed against soluble self antigens are functionally silenced and those directed against multivalent membrane bound self antigen are eliminated (S.B.Hartley et al supra; D.M.Russell et al, supra). Thus, the use of synthetic libraries made by VH, DH, JH or VKJK or VLJL recombination in vitro or its equivalent may be particularly advantageous for isolation of antibodies directed against multivalent membrane bound self antigens.

Applications of antibodies to self antigens

Human antibodies to cell surface components The isolation of such antibody specificities would be particularly useful for preparing agents which mediate cell killing for instance of cancer cells, for example using the natural effector function of antibodies. Anti-self antibodies may also be valuable in the preparation of diagnostic in vivo imaging reagents, for instance using radioisotopes.
Antibodies directed against cell surface components of specific T-cell subsets could be used therapeutically (D.Wraith et al Cell 57 709-715,1989; L.Steinman and R.Mantegazza FASEB J. 4 2726-2731,1990), for instance to prevent T cell action causing rheumatoid arthritis.

Human antibodies modifying the function of self molecules
Antibodies can be isolated which modify the action of self

molecules such as hormones, growth factors and receptors through their binding to a specific epitope on the molecule. Multifunctional proteins may have both desirable and undesirable characteristics, particularly if they are used therapeutically. For instance, the lymphokine TNF (tumour necrosis factor) binds to two different classes of cell receptors- one common on vascular endothelial cells, the other common on tumour cells. A mouse antibody to TNF has been made which prevents TNF from binding to endothelial cell receptors while still allowing it to bind to tumour cells thus allowing attack on the tumours without toxic side effects mediated through endothelial cells (Patent application PCT/AU90/00337). For therapeutic use of antibody modifiers of hormone or growth factor molecules, it would be preferable to have a human antibody specificity isolated directly through selection from a phage library.

Human anti-idiotypes Antibodies directed against the antigen combining sites formed by the variable domains of human antibodies may be directly isolated from phage antibody display libraries. If the original antibody is directed against a hormone or growth factor, the mirror image relationship between antigen and antigen combining sites means that the anti-idiotype may mimic the hormone or growth factor.
Anti-idiotypes may also be useful for the treatment of autoimmune disease. They could be used to bind to circulating autoantibodies. However, it may be preferable to attack directly antibody producing cells, for instance using a bispecific antibody directed against a cell surface marker as well as an anti-idiotype specificity. Alternatively, plasmapheresis could be used to remove circulating antibody and the cells treated directly.

Human antibodies against receptors Human antibodies that bind to receptors, blocking or antagonising ligand function could be selected directly fron a phage library displaying antibodies derived from an unimmunised donor.

Human antibodies to prevent transplant rejection Antibodies directed against the major histocompatibility complex proteins could be used to treat patients following transplants in order to prevent rejection.

TERMINOLOGY

Much of the terminology discussed in this section has been mentioned in the text where appropriate.

Specific Binding Pair

This describes a pair of molecules (each being a member of a specific binding pair) which are naturally derived or synthetically produced. One of the pair of molecules, has an area on its surface, or a cavity which specifically binds to, and is therefore defined as complementary with a particular spatial and polar organisation of the other molecule, so that the pair have the property of binding specifically to each other. Examples of types of specific binding pairs are antigen-antibody, biotin-avidin, hormone-hormone receptor, receptor-ligand, enzyme-substrate, IgG-protein A.

Multimeric Member

This describes a first polypeptide which will associate with at least a second polypeptide, when the polypeptides are expressed in free form and/or on the

surface of a substrate. The substrate may be provided by a bacteriophage. Where there are two associated polypeptides, the associated polypeptide complex is a dimer, where there are three, a trimer etc. The dimer, trimer, multimer etc or the multimeric member may comprise a member of a specific binding pair.

Example multimeric members are heavy domains based on an immunoglobulin molecule, light domains based on an immunoglobulin molecule, T-cell receptor subunits.

Replicable Genetic Display Package (Rgdp)

This describes a biological particle which has genetic information providing the particle with the ability to replicate. The particle can display on its surface at least part of a polypeptide. The polypeptide can be encoded by genetic information native to the particle and/or artificially placed into the particle or an ancestor of it. The displayed polypeptide may be any member of a specific binding pair eg. heavy or light chain domains based on an immunoglobulin molecule, an enzyme or a receptor etc.

The particle may be a virus eg. a bacteriophage such as fd or M13.

Package

This describes a replicable genetic display package in which the particle is displaying a member of a specific binding pair at its surface. The package may be a bacteriophage which displays an antigen binding domain at its surface. This type of package has been called a phage antibody (pAb).

Antibody

This describes an immunoglobulin whether natural or partly or wholly synthetically produced. The term also covers any protein having a binding domain which is homologous to an immunoglobulin binding domain. These proteins can be derived from natural sources, or partly or wholly synthetically produced.

Example antibodies are the immunoglobulin isotypes and the Fab, $F(ab^1)_2$, scFv, Fv, dAb, Fd fragments.

Immunoglobulin Superfamily

This describes a family of polypeptides, the members of which have at least one domain with a structure related to that of the variable or constant domain of immunoglobulin molecules. The domain contains two β-sheets and usually a conserved disulphide bond (see A.F. Williams and A.N. Barclay 1988 Ann. Rev Immunol. 6 381-405).

Example members of an immunoglobulin superfamily are CD4, platelet derived growth factor receptor (PDGFR), intercellular adhesion molecule. (ICAM). Except where the context otherwise dictates, reference to immunoglobulins and immunoglobulin homologs in this application includes members of the immunoglobulin

superfamily and homologs thereof.

Homologs

This term indicates polypeptides having the same or conserved residues at a corresponding position in their primary, secondary or tertiary structure. The term also extends to two or more nucleotide sequences encoding the homologous polypeptides.

Example homologous peptides are the immunoglobulin isotypes.

Functional

In relation to a sbp member displayed on the surface of a rgdp, means that the sbp member is presented in a folded form in which its specific binding domain for its complementary sbp member is the same or closely analogous to its native configuration, whereby it exhibits similar specificity with respect to the complementary sbp member. In this respect, it differs from the peptides of Smith et al, supra, which do not have a definite folded configuration and can assume a variety of configurations determined by the complementary members with which they may be contacted.

Genetically diverse population

In connection with sbp members or polypeptide components thereof, this is referring not only to diversity that can exist in the natural population of cells or organisms, but also diversity that can be created by artificial mutation *in vitro* or *in vivo*.

Mutation *in vitro* may for example, involve random mutagenesis using oligonucleotides having random mutations of the sequence desired to be varied. *In vivo* mutagenesis may for example, use mutator strains of host microorganisms to harbour the DNA (see Example 38 below).

Domain

A domain is a part of a protein that is folded within itself and independently of other parts of the same protein and independently of a complementary binding member.

Folded Unit

This is a specific combination of an α-helix and/or β-strand and/or β-turn structure. Domains and folded units contain structures that bring together amino acids that are not adjacent in the primary structure.

Free Form

This describes the state of a polypeptide which is not displayed by a replicable genetic display package.

Conditionally Defective

This describes a gene which does not express a particular polypeptide under one set of conditions, but expresses it under another set of conditions. An example, is a gene containing an amber mutation expressed in non-suppressing or suppressing hosts respectively.

Alternatively, a gene may express a protein which is

defective under one set of conditions, but not under another set. An example is a gene with a temperature sensitive mutation.

Suppressible Translational Stop Codon

This describes a codon which allows the translation of nucleotide sequences downstream of the codon under one set of conditions, but under another set of conditions translation ends at the codon. Example of suppressible translational stop codons are the amber, ochre and opal codons.

Mutator Strain

This is a host cell which has a genetic defect which causes DNA replicated within it to be mutated with respect to its parent DNA. Example mutator strains are NR9046mutD5 and NR9046 mut T1 (see Example 38).

Helper Phage

This is a phage which is used to infect cells containing a defective phage genome and which functions to complement the defect. The defective phage genome can be a phagemid or a phage with some function encoding gene sequences removed. Examples of helper phages are M13K07, M13K07 gene III no. 3; and phage displaying or encoding a binding molecule fused to a capsid protein.

Vector

This is a DNA molecule, capable of replication in a host organism, into which a gene is inserted to construct a recombinant DNA molecule.

Phage Vector

This is a vector derived by modification of a phage genome, containing an origin of replication for a bacteriophage, but not one for a plasmid.

Phagemid Vector

This is a vector derived by modification of a plasmid genome, containing an origin of replication for a bacteriophage as well as the plasmid origin of replication.

Secreted

This describes a rgdp or molecule that associates with the member of a sbp displayed on the rgdp, in which the sbp member and/or the molecule, have been folded and the package assembled externally to the cellular cytosol.

Repertoire of Rearranged Immunoglobulin Genes

A collection of naturally occurring nucleotides eg DNA sequences which encoded expressed immunoglobulin genes in an animal. The sequences are generated by the *in vivo* rearrangement of eg V, D and J segments for H chains and eg the V and J segments for L chains. Alternatively the sequences may be generated from a cell line immunised *in vitro* and in which the rearrangement in response to immunisation occurs intracellularly.

Library

A collection of nucleotide eg DNA, sequences within

clones.

<u>Repertoire of Artificially Rearranged Immunoglobulin Genes</u>

A collection of nucleotide eg DNA, sequences derived wholly or partly from a source other than the rearranged immunoglobulin sequences from an animal. This may include for example, DNA sequences encoding VH domains by combining unrearranged V segments with D and J segments and DNA sequences encoding VL domains by combining V and J segments.

Part or all of the DNA sequences may be derived by oligonucleotide synthesis.

<u>Secretory Leader Peptide</u>

This is a sequence of amino acids joined to the N-terminal end of a polypeptide and which directs movement of the polypeptide out of the cytosol.

<u>Eluant</u>

This is a solution used to breakdown the linkage between two molecules. The linkage can be a non-covalent or covalent bond(s). The two molecules can be members of a sbp.

<u>Derivative</u>

This is a substance which derived from a polypeptide which is encoded by the DNA within a selected rgdp. The derivative polypeptide may differ from the encoded polypeptide by the addition, deletion, substitution or insertion of amino acids, or by the linkage of other molecules to the encoded polypeptide. These changes may be made at the nucleotide or protein level. For example the encoded polypeptide may be a Fab fragment which is then linked to an Fc tail from another source. Alternatively markers such as enzymes, flouresceins etc may be linked to eg Fab, scFv fragments.

The present invention provides a method for producing a replicable genetic display package or population such rgdps of which method <u>comprises the steps</u> of:

(a binding molecule which is)

a) inserting a nucleotide sequence encoding a member of a specific binding pair and an anti-self antibody,
   within a viral genome;

b) culturing the virus containing said nucleotide sequence, so that said binding molecule is expressed and displayed by the virus at its surface.

The present invention also provides a method for selecting a rgdp specific for a particular epitope which comprises producing a population of such rgdps as <u>described above and the</u> additional step of selecting for said binding molecule by contacting the population with said epitope so that individual rgdps with the desired specificity may bind to said epitope. The method may comprise one or more of the additional steps of: (i) separating any bound rgdps from the epitope; (ii)

which is an anti-self antibody

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which is an anti-self antibody

recovering any separated rgdps and (iii) using the inserted nucleotide sequences from any separated rgdps in a recombinant system to produce the binding molecule separate from virus. The selection step may isolate the nucleotide sequence encoding the binding molecule of desired specificity, by virtue of said binding molecule being expressed in association with the surface of the virus in which said encoding nucleic acid is contained.

The present invention also provides a method of producing a multimeric member of a specific binding pair (sbp), which method comprises:
expressing in a recombinant host organism a first polypeptide chain of said sbp member or a genetically diverse population of said sbp member fused to a component of a secreted replicable genetic display package (rgdp) which thereby displays said polypeptide at the surface of the package, and expressing in a recombinant host organism a second polypeptide chain of said multimer and causing or allowing the polypeptide chains come together to form said multimer as part of said rgdp at least one of said polypeptide chains being expressed from nucleic acid that is capable of being packaged using said component therefor, whereby the genetic material of each said rgdp encodes a said polypeptide chain.
Both said chains may be expressed in the same host organism.

The first and second chains of said multimer may be expressed as separate chains from a single vector containing their respective nucleic acid.

At least one of said polypeptide chains may be expressed from a phage vector.

At least one of said polypeptide chains may be expressed from a phagemid vector, the method including using a helper phage, or a plasmid expressing complementing phage genes, to help package said phagemid genome, and said component of the rgdp is a capsid protein therefor. The capsid protein may be absent, defective or conditionally defective in the helper phage.

The method may comprise introducing a vector capable of expressing said first polypeptide chain, into a host organism which expresses said second polypeptide chain in free form, or introducing a vector capable of expressing said second polypeptide in free form into a host organism which expresses said first polypeptide chain.

Each of the polypeptide chain may be expressed from nucleic acid which is capable of being packaged as a rgdp using said component fusion product, whereby encoding nucleic acid for both said polypeptide chains are packaged in respective rgdps.

The nucleic acid encoding at least one of said first and second polypeptide chains may be obtained from a

derived from an animal which has not been specifically immunised with an immunogen

and an anti-self antibody

library of nucleic acid including nucleic acid encoding said chain or a population of variants of said chain. Both the first and second polypeptide chains may be obtained from respective said libraries of nucleic acid.

a binding molecule which is

The present invention also provides a method of producing a member of a specific binding pair (sbp) from a nucleic acid library including nucleic acid encoding said sbp member or a genetically diverse population of said type of sbp members, which method comprises:

expressing in recombinant host cells polypeptides encoded by said library nucleic acid fused to a component of a secreted replicable genetic display package (rgdp) or in free form for association with a polypeptide component of said sbp member which is expressed as a fusion to said rgdp component so that the rgdp displays said sbp member in functional form at the surface of the package, said library nucleic acid being contained within the host cells in a form that is capable of being packaged using said rgdp component, whereby the genetic material of an rgdp displaying an sbp member contains nucleic acid encoding said sbp member or a polypeptide component thereof.

The nucleotide sequences for the libraries may be derived from eg animal spleen cells or peripheral blood lymphocytes. Alternatively the nucleotide sequence may be derived by the <u>in vitro</u> mutagenesis of an existing antibody coding sequence.

The present invention also provides a method of producing a member of a specific binding pair (sbp) which method comprises:

and an anti-self antibody,

a binding molecule which is

expressing in recombinant host cells nucleic acid encoding said sbp member or a genetically diverse population of said type of sbp member wherein the or each said sbp member or a polypeptide component thereof is expressed as a fusion with a component of a secreted replicable genetic display package (rgdp) which displays said sbp member at the surface of the package, nucleic acid encoding said sbp member or a polypeptide component thereof being contained within the host cell in a form that is capable of being packaged using said rgdp component whereby the genetic material of the rgdp displaying said sbp member encodes said sbp member or a polypeptide component thereof, said host organism being a mutator strain which introduces genetic diversity into the sbp member to produce said mixed population.

The present invention also provides a method of producing a member of a specific binding pair (sbp) which method comprises:

expressing in recombinant host cells nucleic acid

and an anti-self antibody,

a binding molecule which is

encoding said sbp member or a genetically diverse population of said type of sbp member wherein the or each said sbp member or a polypeptide component thereof is expressed as a fusion with a component of a secreted replicable genetic display package (rgdp) which displays said sbp member in functional form at the surface of the package, nucleic acid encoding said sbp member or a polypeptide component thereof being contained within the host cell in a form that is capable of being packaged using said rgdp component whereby the genetic material of the rgdp displaying an sbp member encodes said sbp member or a polypeptide component thereof, said fusions being with bacteriophage capsid protein and the rgdps being formed with said fusions in the absence of said capsid expressed in wild-type form.

a binding molecule which is

The present invention also provides a method of producing a member of a specific binding pair (sbp), which method comprises:

and an anti-self antibody,

expressing in recombinant host cells nucleic acid encoding said sbp member or a genetically diverse population of said type of sbp member or a polypeptide component thereof fused to a component of a secreted replicable genetic display package (rgdp) which displays said sbp member in functional form at the surface of the package, nucleic acid encoding said sbp member or a polypeptide component thereof being contained within the host cell in a form that is capable of being packaged using said rgdp component whereby the genetic material of the rgdp displaying an sbp member or a polypeptide component thereof encodes said sbp member or a polypeptide component thereof, said sbp member or polypeptide component thereof being expressed from a phagemid as a capsid fusion, and a helper phage, or a plasmid expressing complementing phage genes, is used along with said capsid fusions to package the phagemid nucleic acid.

The library or genetically diverse population may be obtained from:

(i) the repertoire of rearranged immunoglobulin genes of an animal not immunised with complementary sbp member,

(ii) a repertoire of artificially rearranged immunoglobulin gene or genes

(iii) a mixture of any of (i), (ii),

The capsid protein may be absent, defective or

conditionally defective in the helper phage.

The host cell may be a mutator strain which introduces genetic diversity into the sbp member nucleic acid.

The sbp member may comprise a domain which is, or is homologous to, an immunoglobulin domain.

The rgdp may be a bacteriophage, the host a bacterium, and said component of the rgdp a capsid protein for the bacterophage. The phage may be a filamentous phage. The phage may be selected from the class I phages fd, M13, f1, If1, lke, ZJ/Z, Ff and the class II phages Xf, Pf1 and Pf3. The phage may be fd or a derivative of fd. The derivative may be tetracycline resistant. The said sbp member or polypeptide chain thereof may be expressed as a fusion with the gene III capsid protein of phage fd or its counterpart in another filamentous phage. The sbp member or polypeptide chain thereof may be inserted in the N-terminal region of the mature capsid protein downstream of a secretory leader peptide. The sequence may be inserted after amino acid +1 of the mature protein. The site for insertion may be flanked by short sequences corresponding to sequences which occur at each end of the nucleic acid to be inserted.

(which is an anti-self antibody,)

The host may be E.coli.

Nucleic acid encoding an sbp member polypeptide may be linked downstream to a viral capsid protein through a suppressible translational stop codon.

The expression end product or derivative thereof may be used to prepare a therapeutic or prophylactic medicament or a diagnostic product.

binding molecule which is a

and an anti-self antibody

The present invention also provides recombinant host cells harbouring a library of nucleic acid fragments comprising fragments encoding a genetically diverse population of a type of member of a specific binding pair (sbp), each sbp member or a polypeptide component thereof being expressed as a fusion with a component of a secretable replicable genetic display package (rgdp), so that said sbp members are displayed on the surface of the rgdps in functional form and the genetic material of the rgdps encode the associated sbp member or a polypeptide component thereof. The type of sbp members may be immunoglobulins or immunoglobulin homologs, a first polypeptide chain of which is expressed as a said fusion with a component of the rgdp and a second polypeptide chain of which is expressed in free form and associates with the fused first polypeptide chain in the rgdp.

and an anti-self antibody,

binding molecule which is a

The present invention also pr vides a phage antibody having the form of a replicable genetic display package displaying on its surface in functional form a member of a specific binding pair or a specific binding domain thereof.

In the above methods, the binding molecule , or a domain that is homologous to an immunoglobulin. The antibody and/or domain may be either naturally derived or synthetic or a combination of both. The domain may be a Fab, scFv, Fv dAb or Fd molecule.

is an anti-self antibody

a binding molecule which is a

and an anti-self antibody,

The present invention also provides rgdps as defined above and members of specific binding pairs obtainable by use of any of the above defined methods. The derivatives may comprise members of the specific binding pairs fused to another molecule such as an enzyme or a Fc tail.

Example 1 shows the determination of affinities and cross-reactivities of antibody fragments isolated from a phage library of the natural immune repertoire.

Example 2 shows the isolation of antibodies directed against human tumour necrosis factor-α from a phage library of scFv fragments.

Example 3 shows the isolation of antibodies binding to human thyroglobulin from a phage library of scFv fragments.

Example 1 Properties of antibody fragments directed against 2-phenyl-5-oxazolone and turkey egg lysozyme isolated from a scFv antibody repertoire derived from an unimmunised human and expressed on phage fd

Antibody scFv fragments directed against 2-phenyl-5-oxazolone (αphOx15) and turkey egg lysozyme (αTEL9, αTEL13, αTEL14 and αTEL16) and bovine serum albumin (αBSA3) were selected from a library of scFv fragments, derived from unimmunised humans, expresssed on the surface of phage fd were isolated as described in example 43 of PCT/GB91/01134.

ELISA

Analysis of phage for binding to phOx:BSA, BSA or lysozyme by ELISA was performed on bacterial supernatants containing phage essentially as described (Clackson, T. *et al.*, (1991), *Nature (London)*, 352, 624-628) with 100 μg/ml phOx:BSA or BSA, or 3 mg/ml turkey egg white lysozyme used for coating. The specificity of isolated clones was checked by ELISA of the soluble scFv fragments using plates coated with 1 mg/ml of an various proteins (hen-egg ovalbumin, hen egg lysozyme, chymotrypsinogen A, cytochrome C, bovine thyroglobulin, glyceraldehyde-3-phosphate dehydrogenase, chicken egg white trypsin inhibitor (Sigma), keyhole limpet haemocyanin (CalBiochem). Binding of soluble scFvs to antigen was detected with the mouse monoclonal antibody 9E10 (1 mg/ml), which recognizes the C-terminal peptide tag (Munro, S. and Pelham, H. R. B. (1986), *Cell*, 46, 291-300) and peroxidase conjugated anti-mouse Fc antibody (Sigma), as in Ward, E. S. *et al.*, (1989), *Nature (London)*, 341, 544-546.

Soluble antibody fragments were readily prepared by growth of *E. coli* HB2151, a non-suppressor strain, carrying the phagemid (Hoogenboom, H. R. *et al.*, (1991), *Nucleic Acids Research*, 19, 4133-4137). Soluble scFvs of αphOx15, αBSA3, αTEL9, αTEL13 and αTEL14 were highly specific in an ELISA to test cross reactivity (Fig. 1). The αTEL16 scFv, isolated from the IgG library, could not be detected in ELISA as a soluble fragment, probably due to its low affinity.

Purification of scFvs and affinity determination

The phOx binding scFv clone 15 (αphOx15) and the TEL binding scFv clone 9 (αTEL9), which gave the strongest ELISA signals, were chosen for affinity determination. Colonies of *E. coli* HB2151, a non-suppressor strain, harbouring the appropriate phagemid were used to innoculate 10 l of 2 x TY containing 100 mg ampicillin/ml and 0.1% glucose. The cultures were grown to an

O.D.$_{600\ nm}$ of 0.9 and expression of soluble scFv induced by the addition of IPTG to a final concentration of 1 mM. Supernatant was concentrated 8 fold by ultrafiltration (Filtron, Flowgen) and 200ml loaded onto a 5 ml column of protein A-Sepharose cross-linked by dimethylpimelidate (Harlow, E. and Lane, D. (1988), *Antibodies - a laboratory manual*, Cold Spring Harbour Laboratory Press) to the monoclonal antibody 9E10 that recognizes the C-terminal peptide tag (Clackson, T. *et al.* (1991), *Nature (London)*, 352, 624-628; Munro, S. and Pelham, H. R. B. (1986), *Cell*, 46, 291-300). The column was washed with 100 ml PBS, 10ml PBS-0.5 M NaCl, 10 ml 0.2M Glycine pH 6.0 and 10 ml 0.2 M glycine pH 5.0. The scFv fragment was eluted with 10 ml 0.2 M glycine, pH 3.0, neutralised with Tris base and dialyzed into PBSE, (PBS buffer containing 0.2 mM EDTA). Supernatant from a separate induction of the αTEL9 was purified on lysozyme-Sepharose (Ward, E. S. *et al.*, (1989), *Nature (London)*, 341, 544-546).

Affinities were measured by fluorescence quench techniques, based on the quenching of tryptophan fluorescence by the bound hapten or antigen (Eisen, H. N., (1964), *Meth. Med. Research*, 10, 115-121; Foote, J. and Milstein, C. (1991), *Nature (London)*, 352, 530-532). All measurements were made with a Perkin-Elmer LS-5B spectrofluorimeter, using an excitation wavelength of 280 nm. Antibody (0.9 ml) in PBSE buffer, was placed in a 4 x 10 mm cuvette in the instrument, and held at 20 °C.

For determination of the affinity of αphOx15, fluorescence quench titration was performed essentially as Foote, J. and Milstein, C. (1991), *Nature (London)*, 352, 530-532. A regime of hapten excess was used: the antibody concentration (100 nM) was at most equal to the lowest concentration of hapten. Negligible volumes of the hapten 4-g-amino-butyric acid methylene 2-phenyl-oxazol-5-one (phOx-GABA) were added to αphOx15 protein to cover a concentration range of 1/5 to 4 times the preliminary estimate of the dissociation constant (500 nM), and the fluorescence determined 1 minute after each addition. Emission was monitored at 340 nm. Data were averaged from 3 runs and the value of the equilibrium constant was obtained from a least-squares fit of the data to a hyperbola.

Fluorescence quench titration was also used t determine the affinity of αTEL9 (Eisen, H. N., (1964), *Meth. Med. Research*, 10, 115-121). αTEL9 protein at 200 nM was titrated to 2-fold molar excess with TEL (Sigma) in PBSE, sample fluorescence being determined 1 minute after each addition. Emission was monitored at 350 nm and the titration repeated six times. Five identical titrations with TEL were also performed on αphOx15 as control. The fluorescence data from each of the six titrations of αTEL9 were subtracted from the mean fluorescence values from the five control titrations of αphOx15 to account for the fluorescence contributed by the added TEL. To obtain the equilibrium constant, fluorescence data, averaged from the six corrected titrations of αTEL9, were fit by least-squares to a hyperbola.

Soluble scFv αTEL9 was purified in one step on a TEL sepharose column or via its c-myc peptide tag on a 9E10 antibody column. Soluble scFv αphOx15 was purified in one step on a 9E10 column. Typical yields were 2 mg/*l* after purification on 9E10 and 5-10 mg/*l* after purification on an antigen column. The dissociation constant of the αTEL9 scFv was 86 nM ± 61 nM and the dissociation constant of the αphOx15 scFv was 534 nM ± 72 nM. The high standard error observed for the dissociation constant of αTEL9 has previously been observed for hen egg lysozyme binding antibodies using this technique.

Finally, soluble αTEL9 scFv could be used to detect lysozyme (1 μg) in a Western blot (data not shown).

Example 2.

Isolation of antibody fragments directed against self antigens from a library of scFvs made from unimmunized blood donors.

Introduction

Naturally occuring V-genes isolated from human PBLs can be constructed into a large library of antibody fragments which contain reactivities against antigens to which the donor has not been exposed (PCT/GB91/01134 ex 42). These libraries may also contain reactivities against self antigens, arising either from self-reactive B-cells which have not been deleted or as non-naturally occuring fragments resulting from VH and VL chain recombination. To test this hypothesis, we panned a large human scFv library displayed on the surface of a phagemid against human TNF-α and a human IgG/κ immunoglobulin.

Methods

Rescue of the library: The library of svFvs was constructed from the RNA of human PBLs and has been previously described (PCT/GB91/01134 ex 42). To rescue phage displaying antibody fragments, approximately 10^9 *E. coli* harbouring the phagemid were used to innoculate 50 ml of 2 x TY containing 1% glucose and 100 μg/ml of ampicillin (2 x TY- AMP-GLU) and grown to an O.D. of 0.8 with shaking. Five ml of this culture was used to innoculate 50 ml of 2 x TY- AMP-GLU, 2 x 10^8 TU of delta gene 3 helper were added and the culture incubated at 37° C for 45 minutes without shaking and then at 37° C for 45 minutes with shaking. The culture was centrifuged at 4000 r.p.m. for 10 min. and the pellet resuspended in 2 litres of of 2 x TY containing 100 μg/ml ampicillin and 50 μg/ml kanamycin and grown overnight. Phage was prepared as previously described (PCT/GB91/01134 ex 44).

Panning of the library: Immunotubes (Nunc) were coated overnight in PBS with 4 ml of either 100 µg/ml or 10 µg/ml of recombinant human TNF-α in PBS or 4 ml of 10 µg/ml of Fog-1, a human IgG/k immunoglobulin which recognizes the human red blood cell Rh (D) antigen. Tubes were blocked with 2% Marvel-PBS for 2 hours at 37° C and then washed 3 times in PBS. Approximately 10^{13} TU of phage was applied to the tube and incubated for 30 minutes at room temperature tumbling on an over and under turntable and then left to stand for another 1.5 hours. Tubes were washed 10 times with PBS 0.1% Tween-20 and 10 times with PBS. Phage were eluted by adding 1 ml of 100 mM triethylamine and rotating 15 minutes on an under and over turntable after which the solution was immediately neutralized with 0.5 ml of 1.0 M Tris-HCl, pH 7.4. Phage were then used to infect 10 ml of mid-log *E. coli* TG1 by incubating eluted phage with bacteria for 30 minutes at 37° C. The E. coli were then plated on TYE plates containing 1% glucose and 100 µg/ml ampicillin. The resulting bacterial library was then rescued with delta gene 3 helper phage as described above to prepare phage for a subsequent round of selection. This process was then repeated for a total of 4 rounds of affinity purification with tube-washing increased to 20 times with PBS, 0.1% Tween-20 and 20 times with PBS for rounds 3 and 4.

Characterization of binders: Eluted phage from the 3rd and 4th rounds of selection were used to infect E. coli HB 2151 and soluble scFv was produced (PCT/KR6A/a134 ex 27) from single colonies for assay. In the case of TNF, phage was also rescued from single colonies. ELISAs were performed as previously described with microtitre plates coated with either 10 µg/ml human TNF-α in 50 mM bicarbonate pH 9.6 or 10 µg/ml Fog-1 in PBS. Clones positive in ELISA were further characterized by PCR fingerprinting (PCT/DB91/01134 ex 20) and then by sequencing.

Results

C 002931

TNF: Soluble scFv from 1536 colonies and phage from 1152 colonies were screened by ELISA and positive clones were further characterized by PCR fingerprinting and sequencing. In this manner, 15 different binders were identified. Four of these have been partially sequenced (Table 1).

Fog-1: Soluble scFv from 96 clones was screened by ELISA and positive clones were further characterized by PCR fingerprinting and sequencing. In this manner, four different binders were identifed (see Table 1).

Table 1

Fog-1 Binders

VH

a-FogB1	QVQLVESGGGLVQPGGSLRLSCAASGFTFS	SYWMS	WVRQAPGKGLEWVA	NOKQDGSEKYYVDSVKG	RFTISRDNAKNTLYLQMNSLRDEDTAVYYCAK	NPRDSGSYYYFDY	WGQG
a-FogA4	LQSGGGVVQPGRSLRLSCAASGFTFS	NYAIH	WVRQAPGKGLEWVA	VISYDGGYEYYADSVKG	RSTISRDNSKNTLYLQMNSLRAEDTAVYYCAR	DASVHTAPYYYMDV	WGK
a-FogG8	LSLTCAVYGGSFS	GYYWIG	WVRQMPGKGLEWMG	IINPGDSDTRYSPSFQG	QVTISVDKSVSTAYLQWSSLKPSDSAVYFCAR	HDVGYCSSPNCAKRPEYFQH	WGQ
a-FogH6	QVQLQQSGGGLVQPGGSLRLSCAASGFTFS	NSGMN	WVRQAPGKGLEWVS	YISSSSSTIYYADSVKG	RFTISRDNAKNSLYLQMNSLRDEDTAVYYCAR	EEGGLMDV	WGKG

VL

a-FogB1	SALTQPASVSGSPGQSITISC	TGTSSDVGGYNYVS	WYQQYPGKAPKLLIY	EVSKRPS	GVSNRFSGSKSGNTASLTISGLQTEDEADYFC	SAYAPTGIMM	FGGGTKLTVLG
a-FogA4	SVGDRVTITIC	RASQGISNYLA	WYQQKPGKVPKLLIY	AASTLQS	GVPSRFSGSGSGTDFTLTISSLQPEDVAVYYC		
a-FogG8		IGLMLA	WYQQKPGKAPKLLIY	AASTLQT	GVPSTFSGSGSGTEFTLQXXXXXPEDFATYYC	QQLISYPLT	FGGGTKVEIKR
a-FogH6	VSSTLSASIGDRVTITC	RASQNIDSWLA	WFQHKPGKAPKPLIY	GAFTLQN	GVPSRFSGSGSGTEFSLTISSLQPDDFATYFC	QQANSFPPT	FGGGTRLEIKR

TNF-a Binders

VH

a-TNFA1	QVQLVESGGGLVQPGGSLRLSCAASGFTFS	SYGMH	WVLQAPGKGLEWVA	FIRYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCVR	EDHVITTGRYHYYMDV	WGK
a-TNFE1	QVQLQESGGGLVQPGGSLRLSCAASGLTFS	SYAMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMDNLRAEDTAVYYCVR	EDYVITSGFYYYNMDV	WGK
a-TNFB4	QLVQSGAEVKKPGASVKVSCKASGYTFS	TYGIS	WVRQAPGQGLEWMG	WISGYNGNTNNAQKLQG	RVSMTTDTSTNTAYMELRSLRSDDTAMYYCAR	DTYSRGYDGLDI	WGQ
a-TNFE7	VQLQESGAEVKKPGSSVKVSCKASGGTFS	SYAIS	WVRQAPGQGLEQMG	GIIPIFGTANYAQKFQG	RVTITADESTSTAYMELSSLRSEDTAVYYCAR	GPLRGYDYYYYYMDV	WGK

VL

a-TNFB4			LVIY	GKNNRPS	GIPDRFSGSSSGNTASLTITGAQAEDEADYYC	NSRDSSGNHLV	FGGGTKLTVL
a-TNFE7	DRVTITC	RGSQGIRNDLG	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYFC	QQANSLPPT	FGGGTK

C 002933

13

Example 3 Isolation of antibody fragments specificities directed against human thyroglobulin from a library of scFv fragments using display on bacteriophage fd

Example 44 of PCT/GB91/01134 describes the selection of antibody scFv fragments directed against thyroglobulin from a library of scFv fragments, derived from unimmunised humans, expresssed on the surface of phage fd were isolated by panning against bovine thyroglobulin. Sixteen clones specific for bovine thyroglobulin were analysed for binding to human thyroglobulin in an ELISA assay as described in example 44 of PCT/GB91/01134. Nine of these clones also bound strongly to human thyroglobulin with absorbance signals of between 1.0 and 1.6 12 minutes after addition of substrate. No cross-reactivity (signal less than 0.05 after 90 min) was found with a panel of unrelated antigens- hen egg lysozyme, BSA, ovalbumin, chymotrypsinogen, cytochrome c, keyhole limpet hemocyanin, insulin, cardiolipin and DNA.

Thus antibodies with specificity for the self antigen, thyroglobulin can be isolated from libraries prepared from unimmunised humans. This procedure using an analogous molecule from another animal to select antibodies and then screening for cross reactivity with the human antigen may be a general approach for the isolation of anti-human antibodies.

Figure 1. Specificity of soluble single chain Fvs (scFvs). Binding was determined by ELISA to a variety of proteins. αTEL9, αTEL13 and αTEL14 = 3 anti-turkey lysozyme scFvs; αphOx15 = anti-2-phenyloxazol-5-one scFv; αBSA-3 = anti-bovine serum albumin scFv. Antigens: TEL (solid box) = turkey egg lysozyme; phOx-BSA (crosshatched box)= 2-phenyloxazol-5-one coupled to bovine serum albumin; BSA (stippled box) = bovine serum albumin; other antigens (open box) = keyhole limpet hemocyanin, bovine thyroglobulin, chymotrypsinogen A, hen-egg ovalbumin, cytochrome c, hen egg lysozyme, chicken egg white trypsin inhibitor, glyceraldehyde-3-phosphate dehydrogenase, and plastic.

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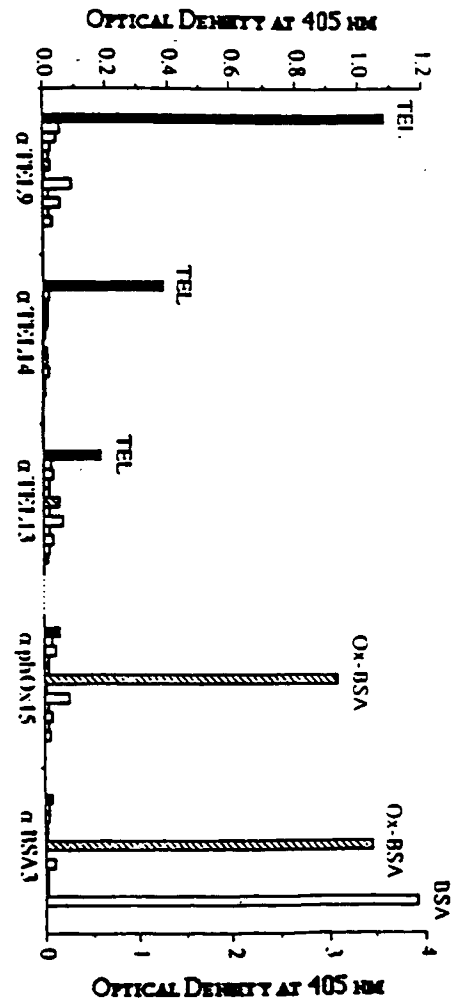

Fig. 1

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